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Assessing the impact of diclofenac, ibuprofen and sildenafil citrate (Viagra®) on the fertilisation biology of broadcast spawning marine invertebrates

Norlaila Mohd Zanuri[$], Matthew G. Bentley[#] & Gary S. Caldwell[*]

School of Marine Science and Technology, Newcastle University, Ridley Building, Claremont Road, Newcastle upon Tyne, NE1 7RU, UK

[*]Corresponding author: gary.caldwell@ncl.ac.uk; +44 (0)191 208 6660

[$]Current address: University Science Malaysia, 11800 Gelugor, Penang, Malaysia

[#]Current address: Newcastle University Singapore, 127A Ang Mo Kio Avenue 8 #05-01, SIT Building@Nanyang Polytechnic, Singapore 567739

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Abstract:

Exposure to synthetic chemicals is a key environmental challenge faced by aquatic organisms. The time and dose effects of the pharmaceuticals diclofenac, ibuprofen, and sildenafil citrate on sperm motility and successful fertilisation are studied using the echinoderms, *Asterias rubens* and *Psammechinus miliaris*, and the polychaete worm *Arenicola marina*, all important components of the marine benthos. Motility was reduced for all species when exposed to diclofenac concentrations ≥ 0.1 µg/L. Exposure to ≥1.0 µg/L of ibuprofen affected only *P. miliaris* gametes and fertilisation success of *A. marina*. *A. rubens* and *P. miliaris* sperm increased in both percentage motility and swimming velocity when exposed to sildenafil citrate at concentrations ≥18 and ≥50 ng/L, respectively. Pre-incubation of sperm with sildenafil citrate significantly increased fertilisation success in *A. rubens* and *P. miliaris* but not in *A. marina*. Pre-incubated *A. rubens* oocytes fertilised successfully in ibuprofen. According to EU

Directive 93/67/EEC, diclofenac is classified as a very toxic substance to gametes of *A. rubens*, *P. miliaris*, and *A. marina* (EC_{50} = 100-1,000 µg/L) while ibuprofen is classified as very toxic to gametes of *P. miliaris* but non-toxic to gametes of *A. marina* (EC_{50} >10,000 µg/L). The present study indicates that diclofenac exposure may have negative impacts on invertebrate reproductive success, whereas ibuprofen potentially may compromise *P. miliaris* reproduction. This study provides a valuable insight into the mechanisms that allow marine invertebrates to survive and reproduce in contaminated and changing habitats.

1. Introduction

Pharmaceutical compounds are a growing class of environmental contaminants within the broad category of pharmaceutical and personal care products (Dietrich et al., 2002). Many pharmaceuticals and their metabolites are detected in wastewater and sewage treatment plants — implicated as the primary sources of environmental discharge (Daughton and Ternes, 1999). Despite most pharmaceuticals being detected in aquatic environments in the nanogram-per-litre (ng/L) to low microgram-per-litre (µg/L) ranges (Fent et al., 2006; Kasprzyk-Hordern et al., 2008; Triebskorn and Hetzenauer, 2012), owing to their high biological and pharmacokinetic activities, risks to aquatic biota cannot be excluded (Jobling et al., 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs) are used primarily for analgesic and anti-inflammatory purposes, acting as non-selective inhibitors for one or both cyclooxygenase enzyme isoforms (COX-1 and -2) by interfering with the inflammatory mediator (Zou et al., 1999). NSAIDs are the most widely taken oral drugs category and have been detected in significant quantities in municipal effluent (Tixier et al., 2003). Diclofenac (2-[(2,6-dichlorophenyl)amino] phenylacetic acid) and ibuprofen ((+/-)-2-(p-isobutylphenyl) propionic acid) are two commonly used NSAIDs, with seawater concentrations ranging between 0.6-843 and 0.01-2,370 ng/L respectively (Ankley et al., 2007; Fent et al., 2006; Gaw et al., 2014). Diclofenac, which has a low removal rate during wastewater treatment, is commonly identified in aquatic ecosystems, with greywater discharge being the principal release pathway (Heberer and Feldmann, 2005). Ibuprofen can remain in the aquatic phase once discharged and is considered fairly persistent, bioaccumulative, of low volatility, and with a low tendency for absorption onto organic matter (Bendz et al., 2005; Fent et al., 2006; Johnson et al., 2007; Maurer et al., 2007; Schwaiger et al., 2004).

Diclofenac and ibuprofen exposure can impact aquatic organisms, including bacteria, algae, molluscs, crustaceans, and teleost fish (Cleuvers, 2003; Dietrich and Prietz, 1999; Ericson et al. 2010; Escher et al., 2005; Han et al., 2006; Heckmann et al., 2007; Hoeger et al., 2005; Seigel et al., 2011); although test concentrations are often higher than those measured in the aquatic environment. Until now, little focus has been directed towards the potential effects on animal reproductive success (Hayashi et al., 2008; Memmert et al., 2013); this translates to inadequate risk assessment.

Sildenafil citrate, also known as Viagra®, is a phosphodiesterase type 5-inhibitor (PDE5 blocker) widely used to treat human male erectile dysfunction (Althof et al., 2006; Glenn et al., 2008) and other conditions requiring the management of pulmonary hypertension (Grant and El-Fakahany, 2004). Sildenafil citrate concentrations in wastewater are detected up to 10 ng/L (Fr. Schröder et al., 2010; Nieto et al., 2010). In contrast to the NSAIDs, the environmental impact of the discharge of sildenafil citrate and its metabolites into aquatic environments is essentially unknown. Rocco et al. (2012) concluded that sildenafil citrate can exert genotoxic effects in teleosts when exposed to 26.25 ng/L for up to 35 days, whilst chronic exposure to mutagenic chlorination derivatives slowed invertebrate population growth (Temussi et al., 2013).

The European Council Directive 2001/83/EC (EC, 2011) concluded an environmental risk assessment should be conducted before authorising marketing of a medicinal product for human use. A two-phased (Phase I and Phase II) tiered assessment concept has been proposed (AMEA, 2005) with Phase I to predict environmental concentrations in surface water (PEC_{sw}). If the PEC_{sw} value is below 0.01 µg/L, and exhibits no environmental concern, no further assessments are required. However, if the PEC_{sw} exceeds 0.01µg/L a Phase II environmental effect analysis is required.

This study assesses the effects of exposure to environmentally realistic concentrations of diclofenac, ibuprofen, and sildenafil citrate over a range of short-term exposure periods on the reproductive success (as measured by sperm motility and fertilisation success) of selected ecologically important benthic marine invertebrates; two echinoderms, sea star *Asterias rubens* (sea star) and *Psammechinus miliaris* (sea urchin), and a polychaete worm *Arenicola marina* (lugworm). The results should allow for a better understanding of the effects of pharmaceutical contaminants on the reproductive success of ecologically important marine invertebrates and potentially permit extrapolations to predict population effects that may inform future risk assessments.

2. Materials and Methods

2.1.Collection and maintenance of animals

Asterias rubens were collected using fishing creels from the Amble coast, Northumberland, UK (55.32 °N, 1.55 °W) from the end of March to early May 2010-2012, were transported in seawater to the laboratory and held in a flow-through seawater aquarium at 5 °C with constant darkness until required. Animals were spawned within one week. *Psammechinus miliaris* were collected in July from two locations on the west coast of Scotland: the Isle of Cumbrae, UK (55.76 °N, 4.94 °W) in 2010 and Oban, UK (56.41 °N, 5.47 °W) during 2011 and 2012. Urchins were transported to the laboratory in tanks filled with ambient seawater and aerated by a portable electric pump. In the laboratory they were held in flow-through tanks at 10 °C and 12L: 12D photoperiod until required. Animals were spawned within one week. *Arenicola marina* were collected by digging during low tide, using a flat pronged fork from beaches at Alnwick, Northumberland, UK (55.38 °N, 1.60°W) during late October to late December, 2010-2012. Once removed from the sand, they were placed into buckets containing small amounts of seawater and sand, and returned to the laboratory where they were sexed by observation of the gametes present in the coelomic cavity under bright illumination (Pacey and Bentley, 1992). Where this was inconclusive, a small drop of coelomic fluid was removed using a disposable syringe fitted with a 21-g hypodermic needle and examined under a light microscope. Following sexing, the animals were kept individually in plastic containers filled with 0.22 µm filtered seawater (FSW), renewed daily, and kept at 5-6 °C. The animals were left for at least 24 hours before spawning to allow gut evacuation.

2.2. Spawning induction, gametes collection and preparation of test solutions

The spawning protocols were followed as published elsewhere; *A. rubens* (Caldwell et al., 2002; Williams and Bentley, 2002); *A. marina* (Pacey and Bentley, 1992); and *P. miliaris* (Caldwell et al., 2004). Gametes were collected in Eppendorf tubes and stored on ice until required.

Ibuprofen (CAS no. 15687-27-1), diclofenac sodium salt (CAS no. 15307-79-6), and sildenafil citrate (CAS no. 171599-83-0) were obtained from Sigma-Aldrich UK, with a chemical purity of 98%. Stock solutions were prepared in high-performance liquid chromatography grade methanol (Sigma-Aldrich, UK). Methanol concentrations did not exceed 0.001% v/v in any experiment. Both ibuprofen and diclofenac were assayed at

concentrations ranging from 0.01 µg/L to a maximum of 1 mg/L, whereas sildenafil citrate was assayed within ranges of 2 ng/L to 1 µg/L. The upper concentration was informed by the tolerance of each bioassay species. Exposure durations of up to one hour were used for *A. rubens* and *P. miliaris* and up to two hours for *A. marina*.

2.3. Sperm motility

Sperm motility was measured using computer assisted sperm analysis (Caldwell et al., 2011). Between six to nine replicate sperm suspensions (1,000 µl) were prepared for each treatment according to the times and concentrations required and mounted on clean, concave glass slides. Sperm motility for each sample was recorded for five seconds in ten fields of view (50-100 sperm per field) providing a total of 60-90 fields for each treatment and time interval. Curvilinear velocity (VCL; µm/s) was assessed, which represents the time-averaged velocity of the sperm head along the actual trajectories of individual spermatozoa.

2.4. Fertilisation success

To test for the effects of oocyte pre-incubation, two hundred and fifty oocytes were incubated in 1 ml of test medium; either solvent control, diclofenac, ibuprofen or sildenafil citrate at set concentrations and times in Eppendorf tubes at 15 ºC. After incubation, the oocytes were washed three times with FSW and transferred into 24-well microplates to which unexposed sperm, pooled from three males, was added to give a final concentration of 2.5 x 10^6 sperm/ml.

The effects of sperm pre-incubation was tested by incubating sperm pooled from three males at a concentration of $5x10^6$ sperm/ml in set concentrations of the solvent control, diclofenac, and ibuprofen, or sildenafil citrate at 15 ºC. The exposed sperm were then added to unfertilised oocytes that were not previously exposed to the test chemicals. After each time point, 250 µl of sperm was added to the unfertilised oocytes to give a final concentration of 2.5 x 10^6 sperm/ml.

A final experiment whereby both oocytes and sperm were separately pre-incubated was conducted using the same conditions as per individual gamete-type pre-incubations. In all fertilisation trials formalin (10% v/v) was added to stop development and preserve the embryos. Fertilisation success was scored by the presence of embryonic cleavage at 60 minutes post fertilisation using a Zeiss inverted microscope.

2.5. Statistical analysis

All statistical analyses were performed using SPSS (v17). Percentage data were arcsine transformed, while log transformation was used for VCL analysis. All data were back transformed for presentation. No-observed-effect (NOEC) and lowest-observed-effect concentrations (LOEC) were determined by analysis of variance (ANOVA) when assumptions for normality and homoscedasticity were met (Shapiro-Wilk and Levene tests, respectively). The significance level was set at $\alpha = 0.05$. Significant ANOVAs were followed by a Dunnett's post hoc test to compare treatment means with control means. Steel's many-one rank test was applied to determine the NOEC or LOEC endpoints if tests for normality and homoscedasticity failed. Later, the Tukey honest significant difference post hoc test was used to identify differences among groups and identify any interaction effect between times and chemical concentrations. Data that did not fulfil normality and homoscedasticity assumptions were subjected to non-parametric Kruskal-Wallis tests followed by a Wilcoxon-Mann-Whitney post hoc test. All figures and tables present the mean ± standard error.

Probit analysis was used to calculate the EC_{50} (half minimal effective concentration) value with 95% confidence limits and fitting the regression equation arithmetically by taking the log of the concentrations used verses the probit value of percentage of immotile sperm and unfertilised oocytes. If the immotile or unfertilised percentage in the control was more than 10% the results with treatment samples were corrected using Abbot's formula (APHA, 1981):

Corrected % = (Pe – Pc)/(100 – Pc) x 100 (Abbott, 1925)

Pe = Experimental percentage

Pc= Control percentage

3. Results

3.1. Sperm motility

The percentage of sperm retaining some motility decreased significantly in *Asterias rubens* when exposed to ≥1 µg/L of diclofenac for periods of 20 minutes and above (Figure 1). Sperm swimming speed (VCL) reduced significantly at a lower diclofenac concentration (0.1 µg/L), with both continuing to decline in a concentration- and time-dependent manner ($F = 2.573$, $p = 0.001$; $F = 2.301$, $p = 0.003$ respectively; supplementary Table 1). A similar, yet more exaggerated pattern was observed for *P. miliaris*, although percentage motility was impacted at 0.1 µg/L after 20 minutes. VCL however reduced almost immediately and, with some variation, continued to decline to less than 50% of controls after 60 minutes of exposure. Both

concentration and exposure period interacted to reduce sperm motility ($F = 7.858$, $p = <0.001$; $F = 1.844$, $p = 0.034$ respectively; supplementary Table 1). Lugworm sperm were exposed for two hours as opposed to a one hour exposure for the echinoderm species. Percentage motility and VCL reduced when exposed to ≥1 µg/L for over 90 minutes, with significant interactions between time and concentration ($F = 1.776$, $p = 0.018$; $F = 3.973$, $p = <0.001$ respectively).

Ibuprofen exposure did not affect any changes in *A. rubens* sperm motility at any concentration of exposure time tested (Figure 2); however, both percentage motility and VCL were reduced in *P. miliaris* at concentrations above 1 µg/L exposed for 20 minutes or more. There was a significant interaction between time and concentration ($F = 28.222$, $p = <0.001$; $F = 2.878$, $p = 0.001$). *A. marina* VCL on the other hand was significantly increased at concentrations above 10 µg/L for incubations of 30 minutes and above, with time and concentration interacting significantly ($F = 3.19$, $p = <0.001$). As this was an increase in overall swimming speed there was no observed reduction in the total percent of sperm remaining motile over the time periods used in this study (Figure 2).

Exposure to sildenafil citrate caused a significant increase in percentage motility for *A. rubens* sperm at concentrations ≥50 ng/L after 40 minutes ($F = 9.712$, $p <0.001$), although VCL increased significantly at concentrations of 18 ng/L and above after 20 minutes, with swimming speeds more than doubling in some treatments (Figure 3). Exposure to ≥50 ng/L was also necessary for a significant increase in *P. miliaris* percentage sperm motility. Curiously, VCL reduced when exposed to 2 and 10 ng/L at 60 minutes, yet increased relative to controls when exposed to concentrations above 50 ng/L; indicating a more complex interaction between *P. miliaris* sperm and sildenafil citrate than observed for the sea star. There were no significant changes in *A. marina* sperm motility or swimming speed.

3.2. Fertilisation success: Pre-incubation of sperm

Incubation of sperm in diclofenac before fertilisation negatively affected fertilisation success for *A. rubens* at concentrations above 1 µg/L when incubated for 20 minutes or more (Figure 4), with fertilisation success dropping to just over 70% after 60 minutes. Interaction between diclofenac concentration and time was significant ($F=1.704$, $p=0.04$). A similar pattern was noted for *P. miliaris* sperm, with sperm incubated for 60 minutes with 1, 10, 100 and 1,000 µg/L having a mean fertilisation success of 72.82, 68.22, 61.33 and 30.88% respectively, whereas controls had a mean fertilisation success of 96.72%. There was a significant interaction between concentration and exposure period ($F = 2.035$, $p = 0.017$). Incubation of sperm before fertilisation in diclofenac also negatively affected the fertilisation

success of *A. marina*. A significant reduction was seen at 30 minutes and 10 µg/L exposure. There was a significant interaction between concentration and time (F = 2.478, p = <0.001).

Pre-incubation of sperm in ibuprofen caused no significant changes in *A. rubens*, with fertilisation success being only affected by time rather than concentration (F = 20.058, p = <0.001). However, *P. miliaris* exhibited a pronounced decline in fertilisation success at 1 µg/L and above, including at time zero (Figure 4). There was a significant interaction between concentration and exposure period (F = 2.738, p = <0.001). Fertilisation success was not significantly affected in the lugworm.

Sildenafil citrate had a positive effect on fertilisation success of *A. rubens* at 50 ng/L and above after 40 minutes of exposure, with a significant interaction between time and concentration (F=6.495, p<0.001; Figure 4). A very similar pattern was observed for *P. miliaris* with a significant interaction between concentration and time (F = 9.868, p = <0.001). There were no significant effects observed for any *A. marina* treatments.

3.3. Fertilisation success: Pre-incubation of oocytes

When *A. rubens* oocytes were pre-incubated with diclofenac there was a significant decrease in fertilisation success at concentrations above 10 µg/L at 20 minutes and above with fertilisation success dropping below 40% in the highest concentration treatment (F = 2.378, p = 0.002). A similar pattern was observed with *P. miliaris*; again with 10 µg/L being the concentration at which significance was determined. As with *A. rubens*, there was a significant interaction between concentration and time (F = 2.427, p = 0.004). For *A. marina*, a significant decline in fertilisation success was also observed at 10 µg/L with a significant interaction between time and concentration (F = 3.741, p = <0.001).

There was no effect of pre-incubation of *A. rubens* or *A. marina* oocytes in ibuprofen. For *P. miliaris*, a significant reduction was only observed when incubated in 100 µg/L for 20 minutes or more. There was a significant interaction between concentration and time (F = 9.858, p = <0.001).

There were no significant differences in any of the sildenafil citrate treatments.

3.4. Fertilisation success: Pre-incubation of both sperm and oocytes

The effects on fertilisation success of pre-incubating both oocytes and sperm are shown in Figure 6. A significant decrease in fertilisation success was observed at diclofenac concentrations of 1 µg/L and above for all three species. A significant reduction was observed when gametes were exposed to ibuprofen at 1 µg/L and above for *P. miliaris* but only at 1,000

µg/L for *A. marina* and there was no impact on *A. rubens* fertilisation success. Pre-incubation with sildenafil citrate above 18 ng/L increased fertilisation success of *A. rubens* and *P. miliaris* ($F = 7.436$, $p = <0.001$; $F = 41.884$, $p = <0.001$ respectively), but had no effect on *A. marina*.

3.5. Toxicity against sperm motility and fertilisation success

Sperm from *A. rubens* were more resistant to ibuprofen and diclofenac than *P. miliaris*, as shown by the 60 minutes sperm motility NOEC (*A. rubens* = 0.10 µg/L, *P. miliaris* = 0.01 µg/L). The NOEC for *A. marina* (0.10 µg/L) could not be determined until after 120 minutes. The EC_{50} (Table 1) for diclofenac effects on sperm motility were 2,335.80 µg/L for *A. rubens*, 378.22 µg/L for *P. miliaris*, 106.77 µg/L for *A. marina*. The 60 minutes ibuprofen sperm motility EC_{50} for *P. miliaris* was 845.98 µg/L. According to the EU Directive 93/67/EEC (EC, 1993) classification there was no toxic effect of ibuprofen against sperm motility of *A. rubens* and *A. marina* as the NOEC was higher than 50 µg/L and 1,000 µg/L respectively. However, ibuprofen is classified as very toxic to *P. miliaris* sperm. Sildenafil citrate is classified as non-toxic for all three species.

Diclofenac is classified as either toxic or very toxic for fertilisation success following sperm pre-incubation; the 60 minutes NOEC for *A. rubens* was 0.1 µg/L and 0.01 µg/L for *P. miliaris*, whereas the 120 minutes NOEC for *A. marina* was 1.0 µg/L. Ibuprofen was non-toxic for *A. rubens* and *A. marina* but very toxic for *P. miliaris*. Sildenafil citrate was non-toxic in all cases. Following oocyte pre-incubation diclofenac was classified as toxic for *A. rubens*, and very toxic for *P. milaris* and *A. marina* (Table 1). Ibuprofen and sildenafil citrate were non-toxic for all three species. When both sperm and oocytes were pre-incubated, diclofenac was classified as very toxic for all three species, whereas ibuprofen was non-toxic for *A. rubens* and *A. marina* but very toxic for *P. miliaris*. Sildenafil citrate was non-toxic in all cases.

4. Discussion

Sperm and oocytes from three ecologically important broadcast spawning marine macroinvertebrates, representing protostome and deuterostome superphyla, were exposed to diclofenac, ibuprofen, and sildenafil citrate (Viagra®) at and above environmentally realistic concentrations. Seawater concentrations have been reported as 0.6-843 ng/L for diclofenac, 0.01-2,370 ng/L for ibuprofen and up to 10 ng/L for sildenafil citrate (Ankley et al., 2007; Fent et al., 2006; Fr. Schröder et al., 2010). Sperm and oocyte responses for each species were different. This in itself is interesting as each species occupies a different trophic level (*A. rubens*

being a predator and scavenger, *P. miliaris* a grazer, and *A. marina* a detritivore), potentially affording each species with differing detoxification capabilities (Sugni et al., 2007). However, it should be noted that while we have assayed the original drug, the main routes of exposure will be through the metabolised for of the drugs, particularly following discharge from sewage treatment plants. Indeed, Mazaleuskaya et al. (2015) show that almost all ibuprofen is metabolised in the human body. To gain a more accurate view of the treats posed by NSAIDs will require further study using the drugs' metabolites. Nonetheless, there is a route to direct discharge of the drugs through disposal down the toilets, for example for drugs that have passed their use-by date (Kuspis and Krenzelok, 1996).

Numerous studies have examined the acute toxicity effects of anti-inflammatory drugs to aquatic organisms. Diclofenac is considered more toxic than other anti-inflammatories such as ibuprofen, naproxen, propranolol, and metoprolol (based on algae, cladoceran, macrophyte and echinoderm studies) (Cleuvers, 2003; Cleuvers, 2004; Láng and Kőhidai, 2012; Ribeiro et al., 2015). This conclusion is validated by the present study. Toxicity appears to relate to the logarithmised octanol-water partitioning coefficient (log K_{ow}), with toxicity increasing with the log K_{ow} (diclofenac = 4.4 whereas ibuprofen = 3.5; although alternative log K_{ow} values have also been reported, e.g. diclofenac = 1.90 and ibuprofen = 2.48; Scheytt et al., 2005); diclofenac therefore being less hydrophilic and presenting a lower bioaccumulation risk factor. However, caution should be exercised when attempting to predict toxicity from hydrophobicity data alone (Puckowski et al., 2016).

Diclofenac reduced sperm swimming speed, the percentage of motile sperm, and fertilisation success with pre-incubated sperm with concentration- and time-dependent relationships. Sperm were more heavily impacted that oocytes. Fertilisation toxicity of diclofenac in *A. rubens* was affected by the additive action of the drug against both gametes; classified as toxic (EC_{50} = 1,679.58-2,335.8 µg/L) to gametes when exposed separately but very toxic (EC_{50} = 616.48 µg/L) when simultaneously exposed. Diclofenac was more toxic to both *P. miliaris* (EC_{50} = 247.31-429.37 µg/L) and *A. marina* (EC_{50} = 106.7-552.34 µg/L) gametes; whereas ibuprofen was non-toxic to *A. rubens* and *A. marina* gametes but very toxic to *P. miliaris* gametes (EC_{50} = 792.96 µg/L). These values are much higher than known seawater concentrations, nevertheless, the NOECs and LOECs fall either within or marginally above environmental concentrations; for instance, exposure of sperm to ibuprofen affected *P. miliaris* with a NOEC of 0.1 µg/L, well within known seawater concentrations. Therefore in

real terms, there exists the potential for harmful impacts on broadcast spawning species although any impact is likely to be minor.

NSAIDs are known as prostaglandin blockers that inhibit the cyclooxygenase enzymes COX-1 and/or COX-2. In humans, these enzymes play important roles to reduce or prevent inflammation. However, the role of COX enzymes in aquatic fauna varies. COX-2 is similar between humans and fish, making fish a potential target of NSAIDs (Zou et al., 1999). It has been suggested that NSAIDs reduce COX activity in molluscs, inducing oxidative stress (Gonzalez-Rey and Bebianno, 2012) and can lead to polyspermic fertilisation in sea urchins (Schuel, 1984). The role of NSAIDs in inhibiting COX enzymes in the studied animals remains unknown, and therefore represents an important knowledge gap to be addressed in a future study.

The present study demonstrates that sildenafil citrate was not toxic to invertebrate gametes. Curiously, *A. rubens* and *P. miliaris* sperm motility was enhanced by sildenafil citrate exposure at concentrations approximating to the upper limits of known environmental levels (Fr. Schröder et al., 2010; Nieto et al., 2010). Of further interest was the observation that this motility enhancement translated into increased fertilisation success in the echinoderms following sperm pre-incubation; although there was no enhancement with oocyte pre-incubation. This improvement in fertilisation success was modulated somewhat when both gametes were pre-exposed. This serves to highlight that sildenafil citrate is acting directly on the sperm as opposed to the oocyte. *A. marina* gametes showed no response to sildenafil citrate exposure. It is worth noting that an increase in sperm motility relative to controls is not necessarily beneficial for the fitness of a species. A spermatozoon carries a finite energy reserve (ATP store). An artificially enhanced swimming speed will exhaust the ATP reserve at an accelerated rate and may shorten the period of sperm longevity. This may ultimately reduce fertilisation success *in situ* where sperm limitation and other limiting factors act upon fertilisation kinetics. The nature of the *in vitro* assays used in our study will have obscured any such effect as sperm were neither limited nor subject to other stressors. It is difficult to predict with any degree of confidence the impacts on fertilisation kinetics in the field as many variables will be at play; however, there would seem to100,000 µg/L be the potential for sildenafil citrate exposure to exert some control on the fertilisation biology of broadcast spawning marine invertebrates.

Sildenafil citrate was first introduced as a phosphodiesterase-5 (PDE5) inhibitor. Cyclic guanosine monophosphate (cGMP) plays an important role regulating sperm motility and

chemoattraction (Darszon et al., 2001; Darszon et al., 2005; Kaupp et al., 2003). The level of cGMP activity is controlled by guanylyl cyclase which is rich in the sperm flagellum (Garbers, 1989; Ward et al., 1985). However, PDE5 works to accept then degrade cGMP which reduces sperm motility. Sildenafil citrate, by blocking PDE5, can increase guanylyl cyclase activity and increase sperm motility in humans and sea urchins (Glenn et al., 2009; Su and Vacquier, 2006).

Fertilisation occurs in the water column in marine broadcast spawners. External stressors, such as pollutants, could affect sperm function (and subsequent fertilisation success) by, amongst other things, the spontaneous generation of reactive oxygen species (Kazama et al., 2014), inducing sperm DNA damage (Lewis and Galloway, 2009), disrupting sperm swimming ability (Caldwell et al., 2004), and the ability of the sperm to undergo the acrosome reaction (Pillai et al., 1997). Sperm limitation and population dynamics are related in broadcast spawners (Levitan and Petersen, 1995). The capacity for motility is crucial if a sperm is to locate and fertilise an oocyte, particularly in situations where sperm density is low (Levitan, 2000).

2.5 Conclusions

We provide additional supporting evidence concerning the risks, albeit minor, that the NSAIDs diclofenac and ibuprofen present to marine fauna, showing significant reductions of sperm motility and fertilisation success within environmentally realistic contaminant levels. A stimulatory effect was observed for sperm motility and fertilisation success in echinoderms in response to sildenafil citrate exposure. This study provides useful data on the toxicity of diclofenac and ibuprofen to marine invertebrate gametes, and as such it makes a valuable contribution to risk assessment of these pharmaceuticals.

Table 1. No-observed-effects (NOEC) or lowest-observed-effects concentration (LOEC), half-minimal effective concentration (EC_{50}), and toxicity substance classification for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Toxicity classification is from EU Directive 93/67/EEC: EC_{50} (µg/L) >100,000 = non-toxic; 10,000-100,000 = harmful; 1,000-10,000 = toxic; <100-1,000 = very toxic; and <100 = extremely toxic. N/A = not applicable as an EC_{50} could not be calculated and is therefore deemed non-toxic.

Species	Test	Pharmaceutical	NOEC or LOEC (µg/L)	EC_{50} (µg/L)	Classification
Asterias rubens	Sperm motility	Diclofenac	NOEC = 0.10	60 min = 2,335.8	Toxic
		Ibuprofen	NOEC = 50.00	N/A	N/A
		Sildenafil citrate	NOEC = 0.18	60 min = 2.25 x 10^{12}	Non-toxic
	Fertilisation: sperm pre-incubation	Diclofenac	NOEC = 0.10	60 min = 2,610	Toxic
		Ibuprofen	NOEC = 50.00	N/A	N/A
		Sildenafil citrate	NOEC = 0.010	60 min= 7.15 x 10^{13}	Non-toxic
	Fertilisation: oocyte pre-incubation	Diclofenac	NOEC = 1.00	60 min = 1,679.59	Toxic
		Ibuprofen	NOEC = 50.00	N/A	N/A
		Sildenafil citrate	NOEC = 0.10	N/A	N/A
	Fertilisation: sperm and oocyte pre-incubation	Diclofenac	LOEC = 0.01	60 min = 616.48	Non-toxic
		Ibuprofen	NOEC = 50.00	N/A	N/A
		Sildenafil citrate	NOEC = 0.01	60 min = 2.37 x 10^{12}	Non-toxic
Psammechinus miliaris	Sperm motility	Diclofenac	NOEC = 0.01	60 min = 378.22	Very toxic
		Ibuprofen	NOEC = 0.1	60 min = 845.98	Very toxic
		Sildenafil citrate	NOEC = 0.018	60 min = 7.23 x 10^{10}	Non-toxic
	Fertilisation: sperm pre-incubation	Diclofenac	NOEC = 0.01	60 min = 298.42	Very toxic
		Ibuprofen	NOEC = 0.10	60 min = 437.03	Very toxic
		Sildenafil citrate	NOEC = 0.10	60min = 6.241 x 10^{10}	Non-toxic
	Fertilisation: oocyte pre-incubation	Diclofenac	NOEC = 1.00	60 min = 429.37	Very toxic
		Ibuprofen	NOEC = 1.00	60 min = 4.56 x 10^{6}	Non-toxic
		Sildenafil citrate	NOEC = 0.01	N/A	N/A
	Fertilisation: sperm and oocyte pre-incubation	Diclofenac	LOEC = 0.01	60 min = 247.31	Very toxic
		Ibuprofen	NOEC = 0.10	60 min = 792.96	Very toxic
		Sildenafil citrate	NOEC = 1.0	N/A	N/A
Arenicola marina	Sperm motility	Diclofenac	NOEC = 0.10	120 min = 106.77	Very toxic
		Ibuprofen	NOEC = 1,000.0	N/A	N/A
		Sildenafil citrate	NOEC = 1.0	N/A	N/A
	Fertilisation: sperm pre-incubation	Diclofenac	NOEC = 1.00	120 min = 565.53	Very toxic

	Ibuprofen	NOEC = 0.10	120 min = 3.24×10^{9}	Non toxic
	Sildenafil citrate	NOEC = 1.0	N/A	N/A
Fertilisation: oocyte pre-incubation	Diclofenac	NOEC = 1.00	120 min = 552.34	Very toxic
	Ibuprofen	NOEC = 100	120 min = 5.16×10^{19}	Non-toxic
	Sildenafil citrate	NOEC = 1.00	N/A	N/A
Fertilisation: sperm and oocyte pre-incubation	Diclofenac	LOEC = 0.01	60 min = 112.61	Very toxic
	Ibuprofen	NOEC = 1.00	60 min = 1.14×10^{19}	Non-toxic
	Sildenafil citrate	NOEC = 1.0	N/A	N/A

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Figure legends

Fig. 1. Percentage of sperm motility (A, C, E) and curvilinear velocity (VCL) (B, D, F) of sperm of *Asterias rubens* (A, B), *Psammechinus miliaris* (C, D), and *Arenicola marina* (E, F) after exposure to diclofenac for set time periods.

Fig. 2. Percentage of sperm motility (A, C, E) and curvilinear velocity (VCL) (B, D, F) of sperm of *Asterias rubens* (A, B), *Psammechinus miliaris* (C, D), and *Arenicola marina* (E, F) after exposure to ibuprofen for set time periods.

Fig. 3. Percentage of sperm motility (A, C, E) and curvilinear velocity (VCL) (B, D, F) of sperm of *Asterias rubens* (A, B), *Psammechinus miliaris* (C, D), and *Arenicola marina* (E, F) after exposure to sildenafil citrate for set time periods.

Fig. 4. Fertilisation success (%) of oocytes from *Asterias rubens* (A-C), *Psammechinus miliaris* (D-F), and *Arenicola marina* (G-I) after incubation of sperm with diclofenac (A, D, G), ibuprofen (B, E, H), and sildenafil citrate (C, F, I) at different concentrations and times prior to fertilisation.

Fig. 5. Fertilisation success (%) of oocytes from *Asterias rubens* (A-C), *Psammechinus miliaris* (D-F), and *Arenicola marina* (G-I) after incubation of oocytes with diclofenac (A, D, G), ibuprofen (B, E, H), and sildenafil citrate (C, F, I) at different concentrations and times prior to fertilisation.

Fig. 6. Fertilisation success (%) of oocytes from *Asterias rubens* (A-C), *Psammechinus miliaris* (D-F), and *Arenicola marina* (G-I) after pre-incubation of oocytes and sperm with

diclofenac (A, D, G), ibuprofen (B, E, H), and sildenafil citrate (C, F, I) at different concentrations and times prior to fertilisation.

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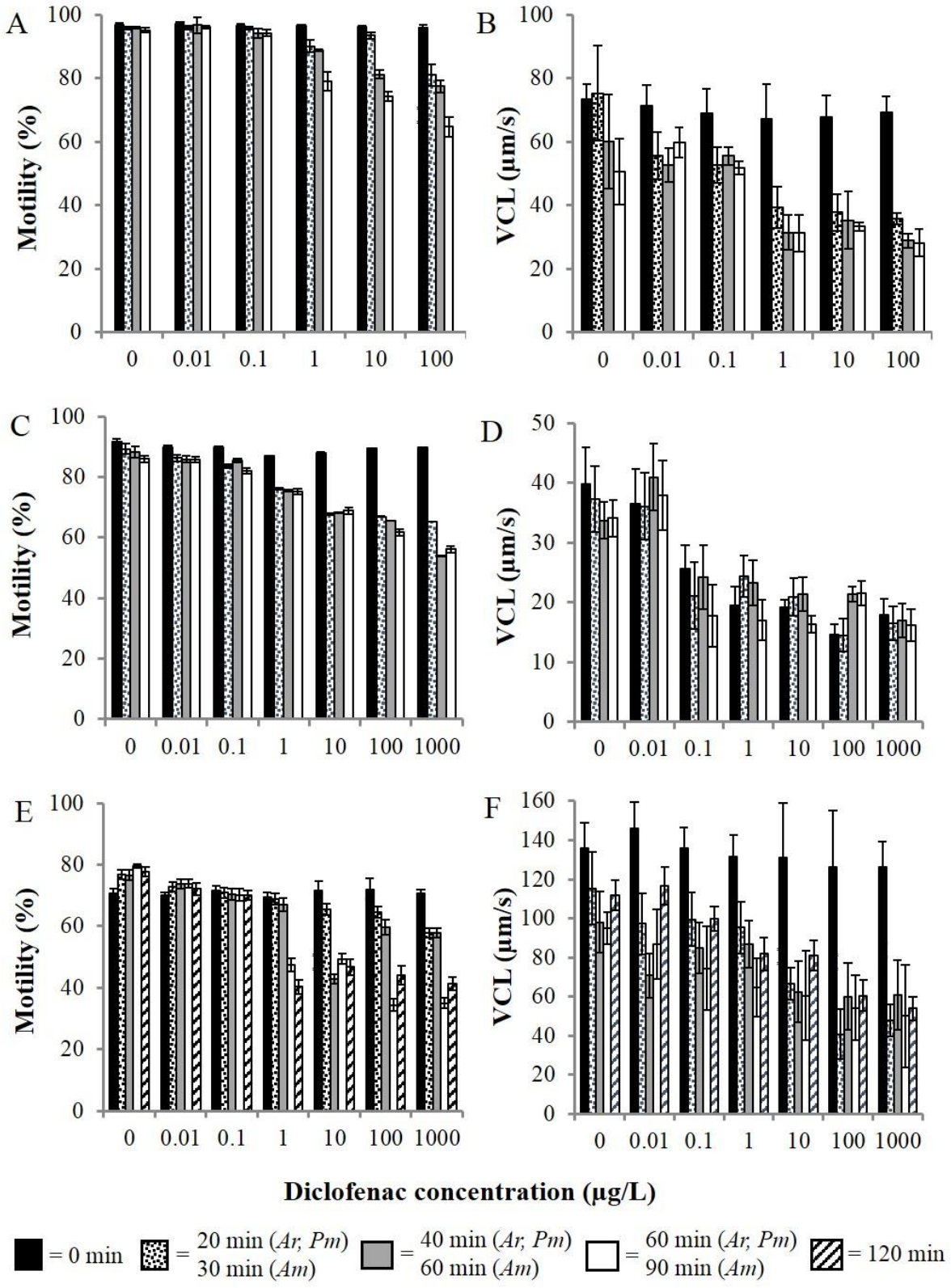
A
B
C
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E
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Motility (%)
VCL (µm/s)
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1000
Diclofenac concentration (µg/L)
= 0 min
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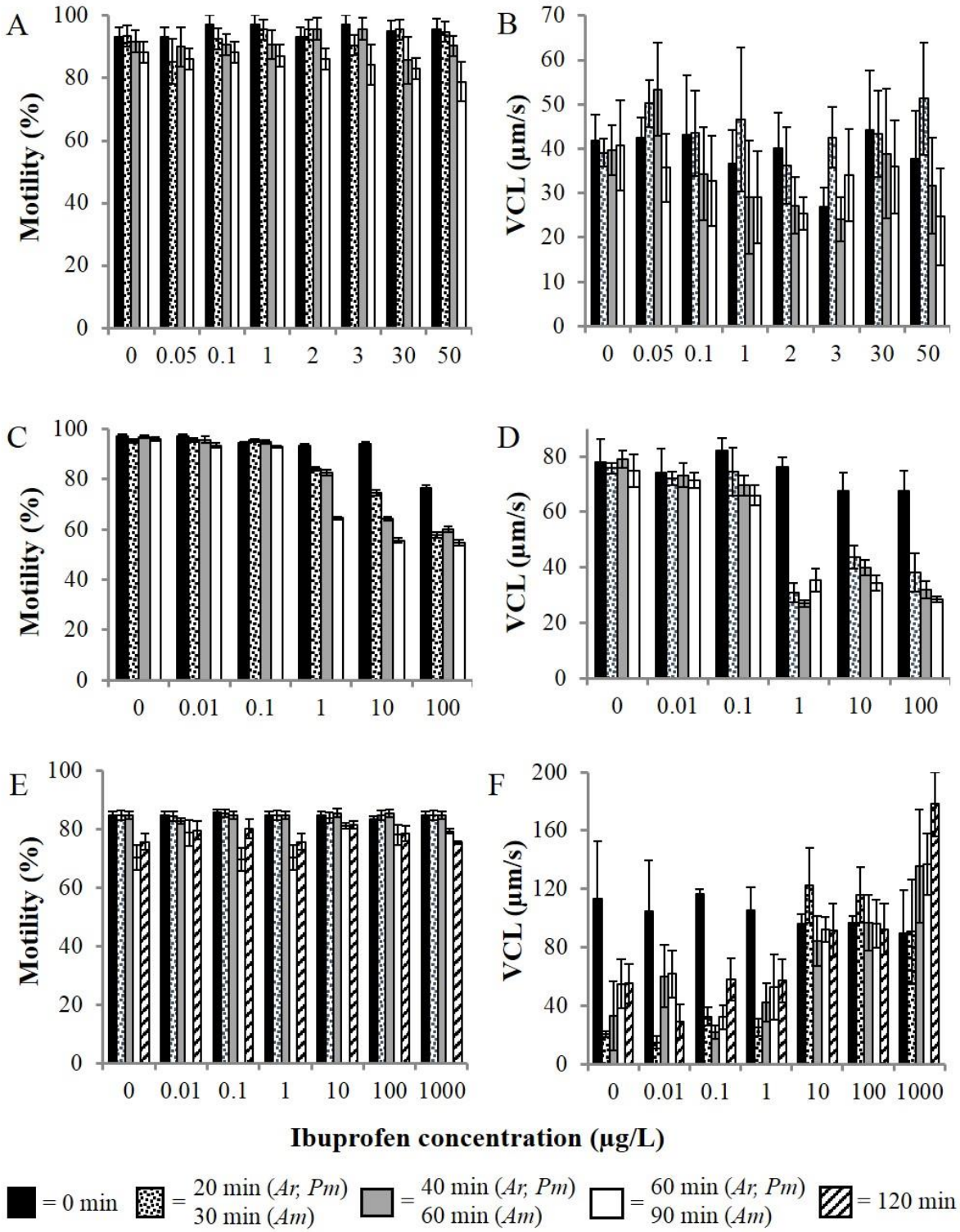
= 40 min (Ar, Pm) 60 min (Am)
= 60 min (Ar, Pm) 90 min (Am)
= 120 min

Fig. 1.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Motility (%)
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VCL (µm/s)
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40
30
20
10
0
0 0.05 0.1 1 2 3 30 50
C
Motility (%)
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80
60
40
20
0
0 0.01 0.1 1 10 100
D
VCL (µm/s)
80
60
40
20
0
0 0.01 0.1 1 10 100
E
Motility (%)
100
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60
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20
0
0 0.01 0.1 1 10 100 1000
F
VCL (µm/s)
200
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Ibuprofen concentration (µg/L)
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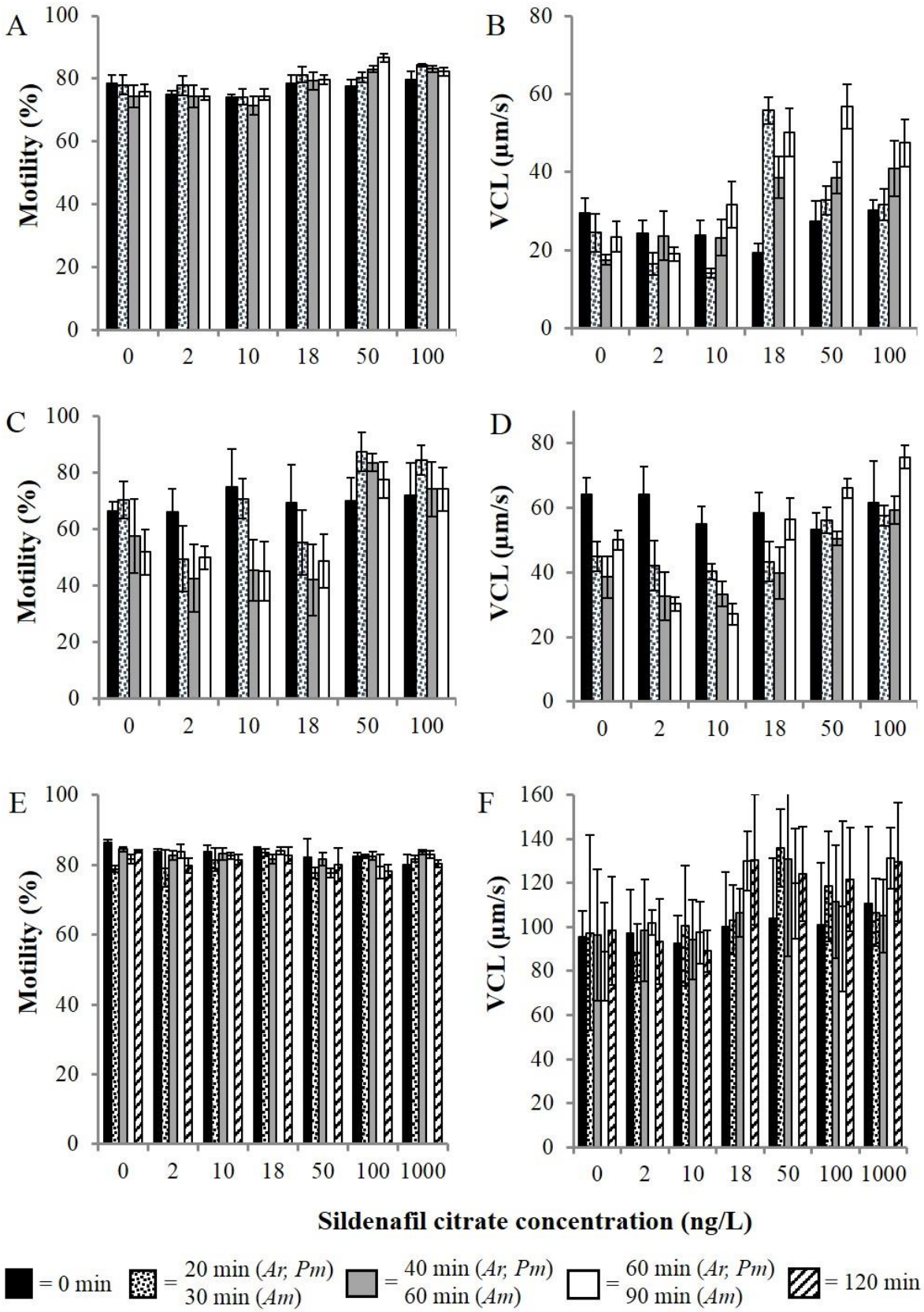
= 120 min

Fig. 2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Motility (%)
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VCL (µm/s)
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Sildenafil citrate concentration (ng/L)
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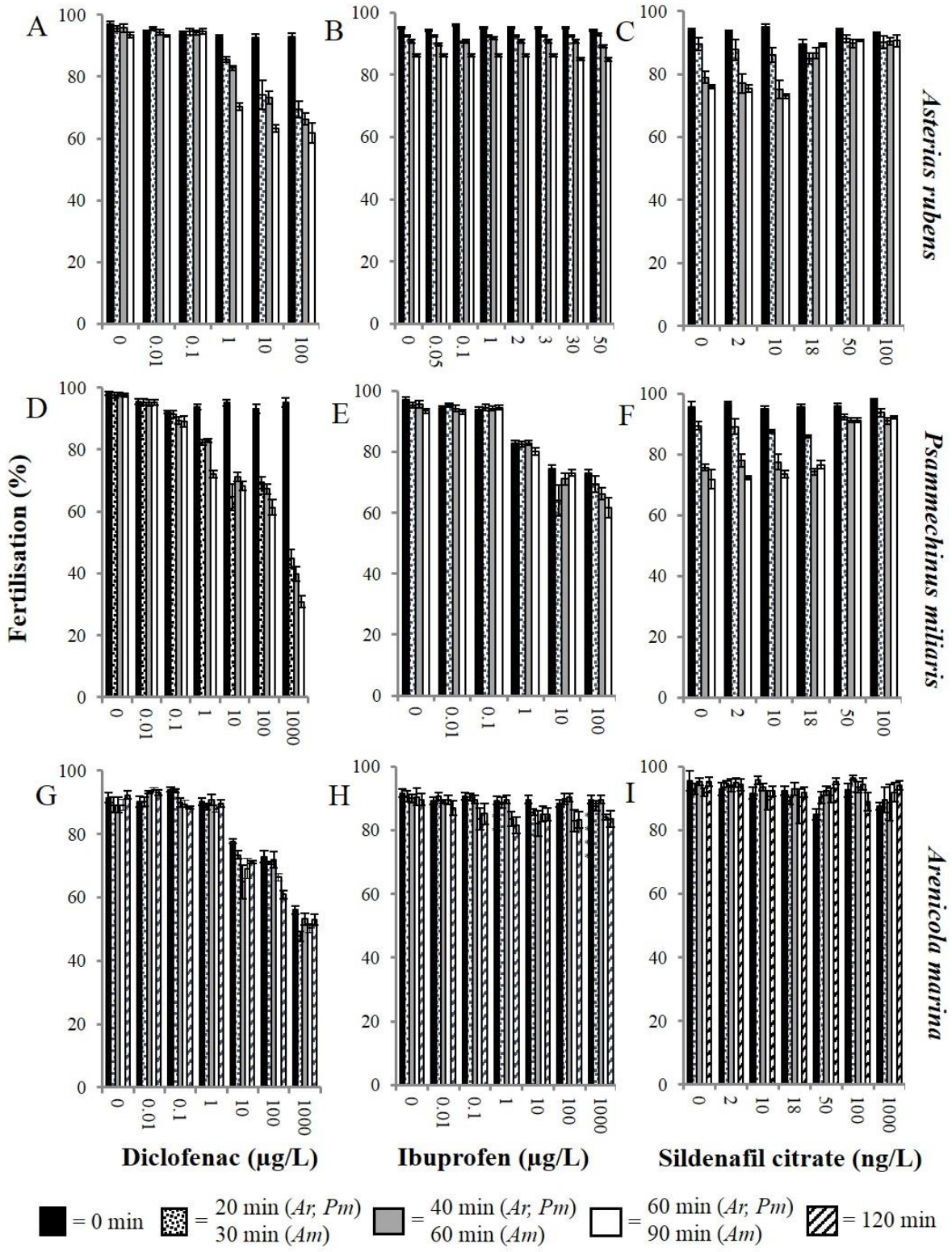
Fig. 3


A
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Asterias rubens
Psammechinus miliaris
Arenicola marina
Fertilisation (%)
Diclofenac (µg/L)
Ibuprofen (µg/L)
Sildenafil citrate (ng/L)
= 0 min
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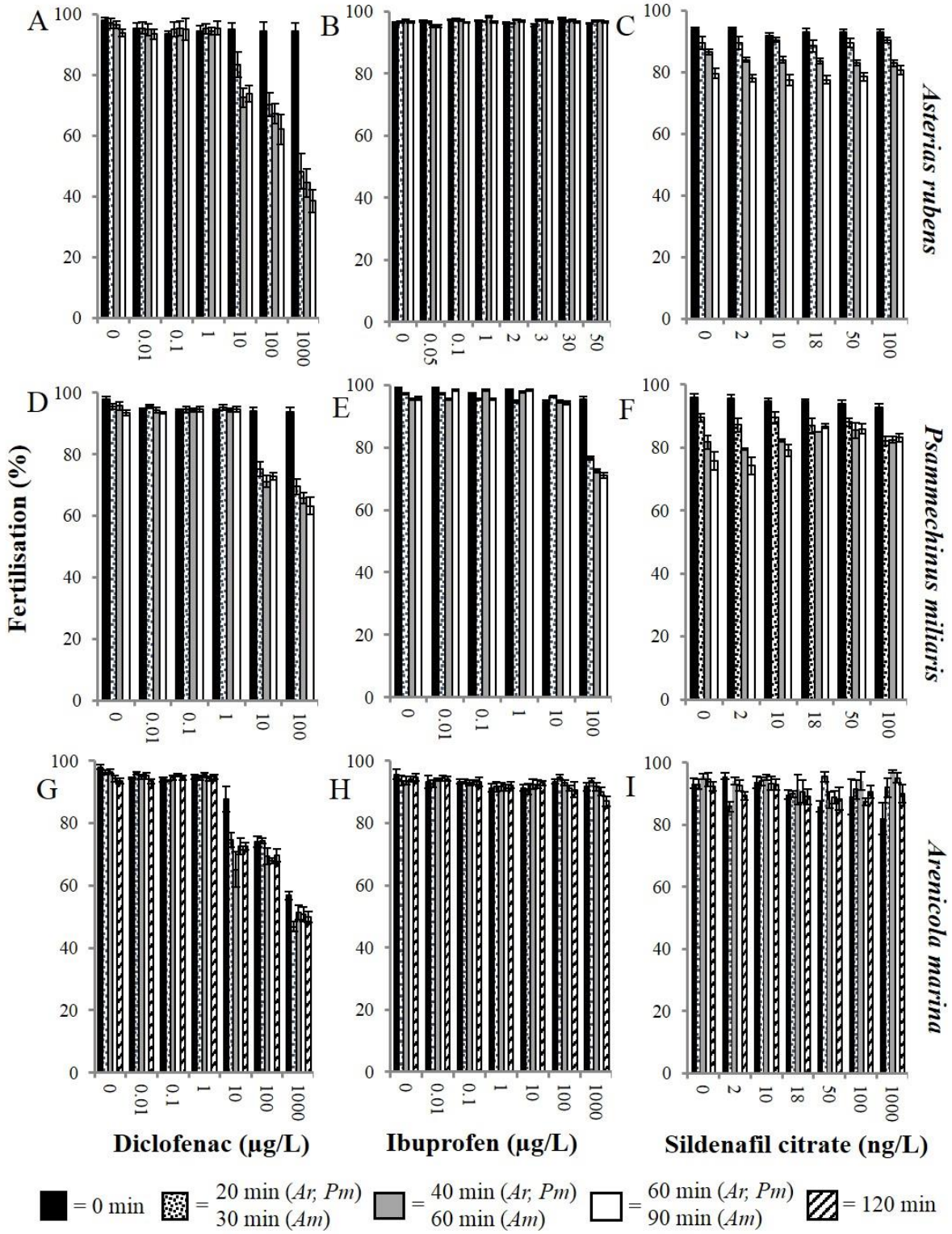
= 60 min (Ar, Pm) 90 min (Am)
= 120 min

Fig. 4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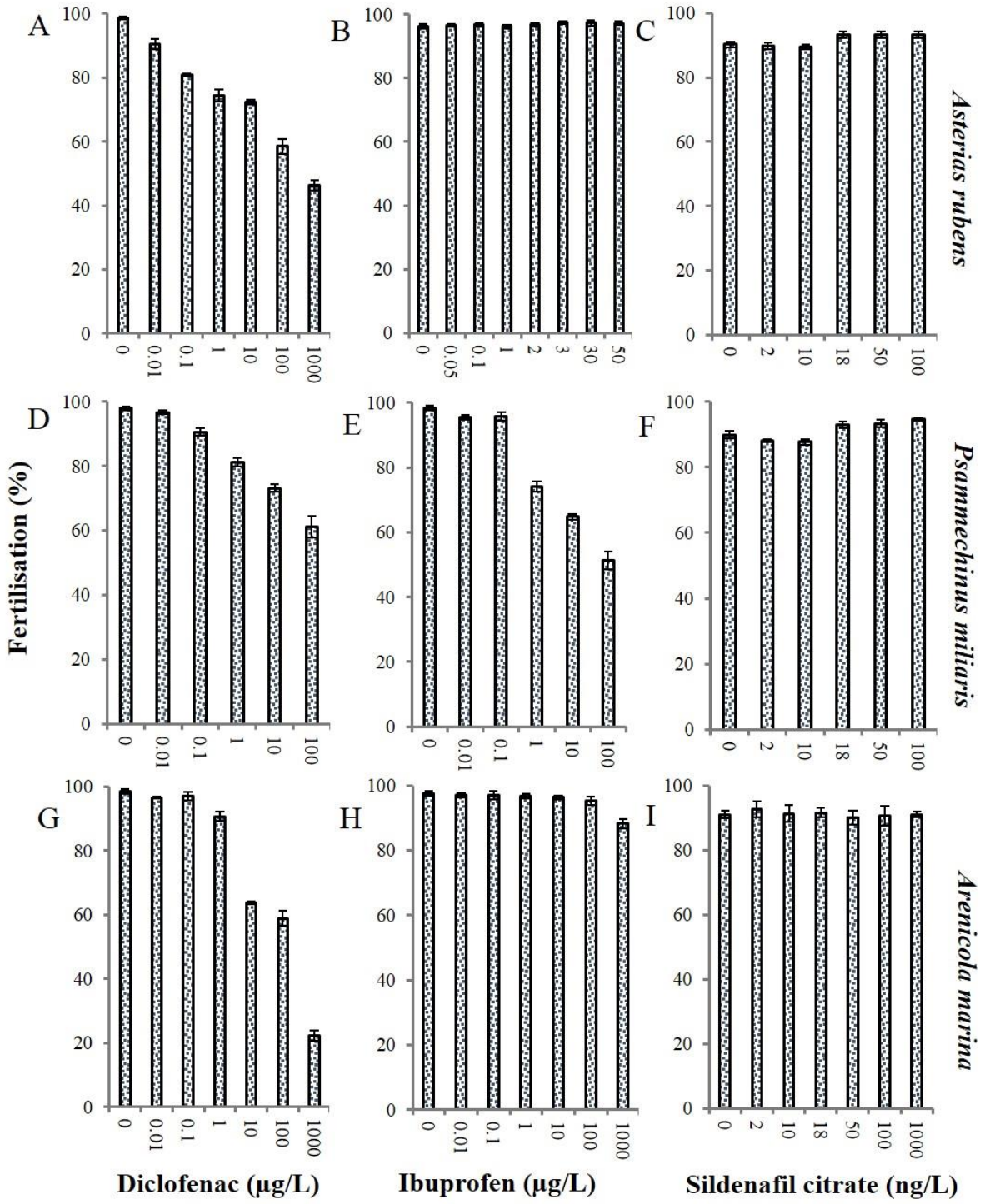
Asterias rubens
Psammechinus miliaris
Arenicola marina
Fertilisation (%)
Diclofenac (µg/L)
Ibuprofen (µg/L)
Sildenafil citrate (ng/L)
= 0 min
= 20 min (Ar, Pm) 30 min (Am)
= 40 min (Ar, Pm) 60 min (Am)
= 60 min (Ar, Pm) 90 min (Am)
= 120 min

Fig. 5


A
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Asterias rubens
Psammechinus miliaris
Arenicola marina
Fertilisation (%)
Diclofenac (µg/L)
Ibuprofen (µg/L)
Sildenafil citrate (ng/L)

Fig. 6